Peroral gene therapy of lactose intolerance using an adeno-associated virus vector

MATTHEW J. DURING[1,2,3], RUIAN XU[1], DEBORAH YOUNG[1], MICHAEL G. KAPLITT[4], ROBERT S. SHERWIN[3] & PAOLA LEONE[2,3]

[1]*Department of Molecular Medicine, University of Auckland School of Medicine, 85 Park Road, Auckland, New Zealand*
[2]*CNS Gene Therapy Center, Department of Neurosurgery, Jefferson Medical College, 1025 Walnut Street, Philadelphia, Pennsylvania 19107 USA*
[3]*Departments of Internal Medicine and Neurosurgery, Yale University School of Medicine, 333 Cedar Street, New Haven, Connecticut 06520 USA*
[4]*Department of Neurobiology, The Rockefeller University, 1230 York Avenue, New York, New York 10021 USA*
Correspondence should be addressed to M.J.D.; email: Matthew.During@mail.tju.edu

Gene therapy is usually reserved for severe and medically refractory disorders because of the toxicity, potential long-term risks and invasiveness of most gene transfer protocols. Here we show that an orally administered adeno-associated viral vector leads to persistent expression of a β-galactosidase transgene in both gut epithelial and lamina propria cells, and that this approach results in long-term phenotypic recovery in an animal model of lactose intolerance. A gene 'pill' associated with highly efficient and stable gene expression might be a practical and cost-effective strategy for even relatively mild disorders, such as lactase deficiency.

Adult-type lactase deficiency is an autosomal recessive condition[1,2] and is the world's most common genetic disorder, afflicting more than 50% of the world's population. Its prevalence ranges from 100% in some Southeast Asian populations to less than 5% in some Northern European countries[3]. Although the symptoms associated with lactose intolerance are relatively mild and readily controlled by omitting lactose-containing foods from the diet, there is some concern for the potential clinical impact of the dietary restrictions that typically accompany lactose intolerance. Specifically, the reduction in calcium intake generally associated with lactose-free diets may reduce bone density and promote osteoporosis and fractures in postmenopausal women and the elderly[4-6],and reduce the bone mineral mass in children[7]. Moreover, in many Asian and African communities, lactose intolerance is associated with consumption of a reduced food repertoire and with malnutrition.

Lactose intolerance is most commonly associated with a reduction in intestinal lactase activity. Lactose digestion is dependent on the enzyme lactase-phlorizin hydrolase (LPH), a microvillar protein that has at least three enzyme activities: galactosidase, phlorizin hydrolase and glycosylceramidase[8]. However, dietary administration of yeast or bacterial β-galactosidase is sufficient to confer the ability to metabolize lactose[9]. Most mammalian species are relatively lactase-deficient after weaning. This developmental change in LPH expression seems to be mostly secondary to a reduction in gene transcription[10-12]. However, there may also be a post-translational component to the reduction in LPH activity[13,14].

Using gene therapy to correct lactase deficiency has not been explored, but it is potentially valuable as it might be done with an orally delivered viral vector. Among the viral vectors that could be used for this purpose, adeno-associated virus (AAV) has several features that make it particularly useful for gene therapy. It is a defective, helper-dependent virus, and wild-type AAV is nonpathogenic in humans and other species[15]. Vectors can be generated that are completely free of helper virus[16]. Recombinant AAV vectors, with the entire coding sequences removed, retain only 145-base-pair terminal repeats. These vectors, therefore, are devoid of all viral genes, minimizing any possibility of recombination and viral gene expression. Moreover, although AAV may induce immunological responses, these are relatively mild compared with the inflammation that accompanies early-generation adenoviral vectors[17]. Another feature of AAV that makes it particularly suitable as an orally-delivered vector is its hardiness: AAV is resistant to temperature and pH extremes and solvents[15]. Furthermore, during active infection in humans, wild-type AAV is typically found in both respiratory and gastrointestinal tract secretions; thus, the gut is a normal host tissue for the virus[15]. Finally, AAV vectors can produce long-term gene expression in terminally differentiated cells in the brain after *in vivo* administration[18].

We chose the rat model of adult-onset hypolactasia, for our study of increasing gastrointestinal expression of β-galactosidase using a viral vector. However, after screening both adult (more than 4 months old) Fisher 344 rats or Sprague-Dawley rats as well as retired breeders (more than 14 months old), we found that most (about 70%) had some level of persistent lactase activity, as determined by a rise in plasma glucose after an oral lactose challenge test. We therefore screened rats in order to select a subgroup that had a flat plasma glucose curve after feeding with lactose and were by this definition lactase-deficient. These rats were randomly selected to receive AAVlac (a recombinant AAV vector expressing β-galactosidase[18]) or PBS vehicle. The vector or vehicle was delivered to fasting rats through an oro-gastric tube.

EXHIBIT "B"

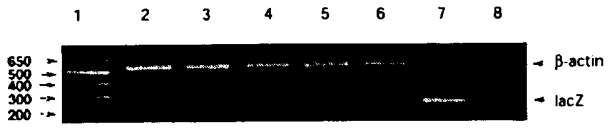

Fig. 1 RT–PCR analysis of total RNA extracted from the proximal intestine from a rat at 3 days and at 6 months after peroral delivery of AAVlac. Lane 1, DNA base-pair ladder (sizes indicated along left margin); lanes 2 and 3, mRNA extracted from rats treated with AAVlac at 3 days and at 6 months, respectively; lanes 4, 5 and 6, mRNA extracts from lungs, gonads and kidneys of a rat treated with AAVlac, at 3 days after treatment; lane 7, positive control (lacZ mRNA extracted from *E. coli*); lane 8, negative control (no reverse transcriptase). Amplification of the lacZ cDNA with this set of primers should generate a 219-bp fragment, consistent with that found for the positive control and lanes 1 and 2 (lower arrow). β-actin cDNA served as an internal control for the PCR reactions (upper arrow).

Rats were allowed to recover and given a regular rat chow diet. At 1 week and again at 4 months after AAVlac administration, rats were challenged with an oral lactose load, and their plasma glucose samples were measured. Rats were also fed a lactose-only diet for 2 weeks after the lactose challenge test at both time points (1 week and 4 months after treatment). Body weights were monitored immediately before lactose feeding began as well as on days 7 and 14 of the diet. These rats were analyzed at 6 months after gene transfer. Other groups of rats were used for additional analyses of gene and protein expression; these included rats dosed with AAVlac or the control vector AAVluc (expressing luciferase). These rats were studied at 3 hours, 6 hours, 3 days and at 1 month after treatment.

AAV vector transduction of gut

In a subgroup of rats, AAVlac mRNA expression was determined using RT-PCR at 3 days and again at 6 months after administration of vector or PBS and control vector (AAVluc). Of those tested, none of the PBS control rats (n = 4) or rats treated with AAVluc (n = 4) had a positive RT-PCR signal, whereas all those treated with AAVlac were positive for lacZ mRNA (n = 4). Moreover, analysis of other organs using PCR showed the vector DNA did not disseminate beyond the gastrointestinal tract (Fig. 1). Expression of the AAVlac protein product *E. coli* β-galactosidase in the gastrointestinal tract was determined using X-gal histochemistry in conditions specific for the bacterial enzyme that do not show endogenous mammalian β-galactosidase activity. Rats underwent X-gal histological analysis at many timepoints (n = 7 at 6 months; n = 4 at 1 month; n = 15 at 3 days; n = 12 at 6 hours; and n = 4 at 3 hours). No X-gal staining was found in the gastrointestinal tract in the first 3 hours after AAVlac administration. However, within 6 hours after treatment, clear blue (X-gal positive) cells were seen in a characteristic distribution with strongest staining in the lamina propria (Fig. 2). We counted cells positive for X-gal to generate quantitative data on protein expression and the transduction efficiency of the oral administration of AAVlac. Within 6 hours after treatment, 18% of lamina propria cells were transduced; this remained stable with 19% at 3 days and was still persistent at 17–19% at 1 and 6 months after treatment. The total number of lamina propria cells transduced ranged from 8 million to 13 million at each time point. This represents a transduction efficiency of about 10% based on the dose of AAV vector administered. High-power magnification of the gut sections showed faint X-gal staining in the epithelial cells. This was confirmed with immunohistochemistry with a monoclonal antibody specific to the *E. coli* β-galactosidase followed by fluorescence detection. At 3 days after vector administration, there was localization of the enzyme in the lamina propria and on the luminal surface of the epithelial cells in the intestinal brush-border in most cells (Fig. 3). By 1 month after treatment, the total number of epithelial cells immunoreactive for *E. coli* β-galactosidase was reduced (over 90%), although β-galactosidase immunofluorescence was obtained at this timepoint, with the protein present throughout the cell (Fig. 4). The highest levels of expression were in the stomach, duodenum and proximal jejunum. No staining was observed in the large bowel. In rats treated with the control recombinant AAV vector AAVluc, X-gal-positive cells and cells immunofluorescent for β-galactosidase were not observed (Figs. 2,3 and 4).

Effect on weight and lactose metabolism

Administration of AAVlac did not affect the weight-gain or behavior of any rats fed regular rat chow. However, both groups, rats treated with AAVlac (n = 7) and those treated with PBS (n = 7), lost weight when fed a lactose-only diet beginning one week after oral vector administation (Fig. 5*b*). During the first week of this diet, this weight loss was similar in both groups, reflecting reduced food intake. However, in the second week, the groups were easily distinguishable in their weight loss: The rats treated with AAVlac had no further weight loss, whereas the rats treated with PBS continued to lose weight at the same rate as during the first week. At 2 weeks after beginning the lactose-only diet, the AAVlac group weighed 314 ± 6 grams, whereas the rats treated with PBS rats weighed 264 ± 6 grams (P < 0.001). Moreover, after an acute lactose challenge on day 7, immediately before beginning the lactose diet, the rats treated with AAVlac had a significant elevation in plasma glucose from 114 ± 4 mg/dl to 130 ± 3 mg/dl (P = 0.013), whereas rats treated with PBS had an essentially flat plasma glucose curve (Fig. 5*a*).

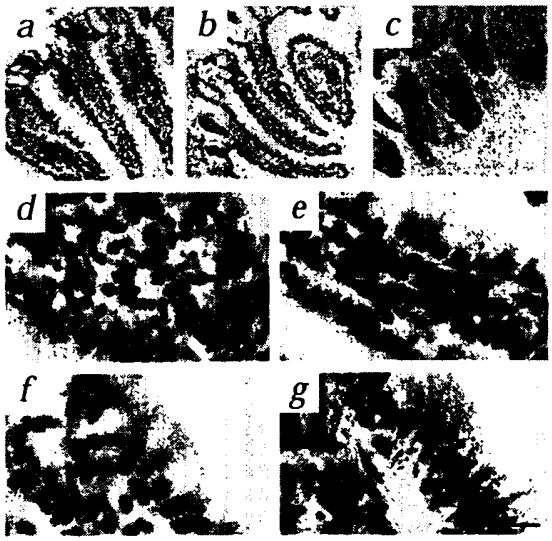

Fig. 2 X-gal staining in gut sections of a control rat treated with AAVluc (***a***) and a rat treated with AAVlac after 3 days (***b***) and 6 months (***e***); images at low magnification. Lamina propria (***d*** and ***e***) and epithelial layer (***f*** and ***g***) from control rats (*d* and *f*) and rats treated with AAVlac (*e* and *g*) show X-gal positive cells in the lamina propria and light staining at the epithelial brush border (*g*) that was not evident in controls; images at high magnification. Scale bar represents 200 μm for *a–c* and 33 μm for *d–g*.

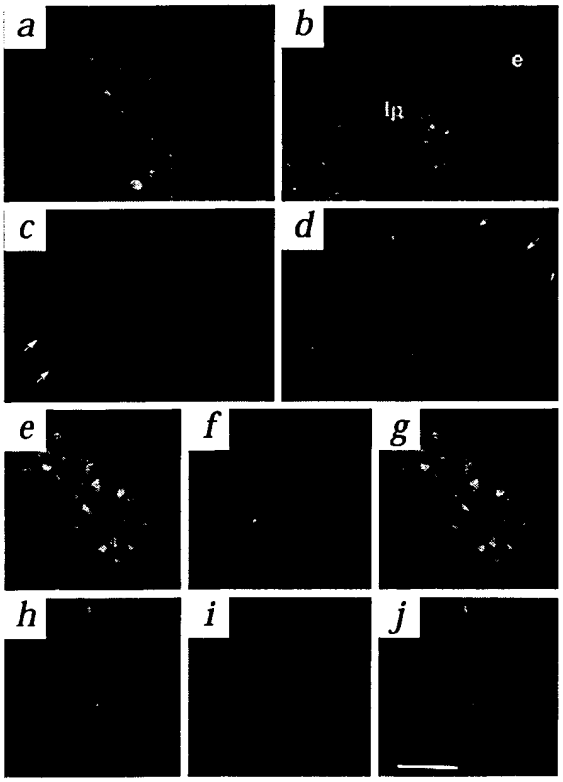

Fig. 3 Propidium iodide staining (*a* and *b*) combined with β-galactosidase immunohistochemistry (*e* and *d*) showing the lamina propria (lp) and epithelial layers (e) from a control rat (treated with AAVluc) (*a*) and a rat treated with AAVlac (*b*) 3 days after vector administration. Many cells immunoreactive with β-galactosidase within the lamina propria and a band of immunoreactivity at the epithelial brush border were found in sections from the rat administered AAVlac (*d*) and not in the control rat (*c*). Images (high magnification) of the lamina propria (*e–g*) and epithelial layer (*h–j*) shown in (*b*), with combined labels in (*g*) and (*j*). Arrows point to the luminal border of the epithelial cells. Scale bar represents 43 μm in *a–d* and 25 μm in *e–j*.

Long-term phenotypic effects

Groups of rats (n = 7) were studied for 6 months after a single oral treatment with the vector. Rats treated with AAVlac behaved normally and gained weight at a rate indistinguishable from that of the PBS controls. At 4 months after vector administration, these rats were re-challenged with a lactose load and then fed a lactose-only diet again. As with the challenge during the first week after administration, the rats treated with AAVlac had a rise in plasma glucose, from 115 ± 4 mg/dl to 127 ± 4 mg/dl (P = 0.03), that did not occur in the controls (Fig 6*a*). Furthermore, the rats treated with PBS had persistent weight loss on the lactose diet, whereas the rats treated with AAVlac, as before, were able to maintain body weight during the second week; the final, 14-day weights were 496 ± 10 grams and 441 ± 9 grams in the groups treated with AAVlac and PBS, respectively (P = 0.002; Fig. 6*b*).

Discussion

This study demonstrates the feasibility of administering an AAV vector orally to obtain long-term gene expression in the gastrointestinal system. There was persistent and stable expression evident for 6 months, and phenotypic correction could be shown for at least 4 months. In normal rats, intestinal LPH expression occurs in the enterocytes with the protein transported to the brush border. The expression of β-galactosidase in our study differed in that it was most apparent in the lamina propria; however, expression was also observed in epithelial cells, with high-powered immunofluorescence confocal microscopy showing localization of the protein at the brush border, consistent with the phenotypic effects observed. As turnover of enterocytes occurs every 3–5 days, the expression we observed at 1 month indicates that progenitor cells lying within the crypts were transduced. The almost complete transduction of proximal intestinal epithelial cells at 3 days is consistent with progenitor cell transduction. As early as 6 hours after AAVlac administration, high levels of gene expression were observed within the lamina propria, a finding consistent with the function of M cells in the gut. M cells are specialized gut epithelial cells scattered throughout the intestine, but most concentrated overlying Peyers patches and clusters of immune cells[19], that scavenge foreign proteins, viruses and bacteria, and rapidly (within 3 hours) transport these foreign agents to the immune cells within the lamina propria. The early expression of vector encoded β-galactosidase within the lamina propria is consistent with this pathway. The main phenotypic effect of AAVlac administration here was the rats'

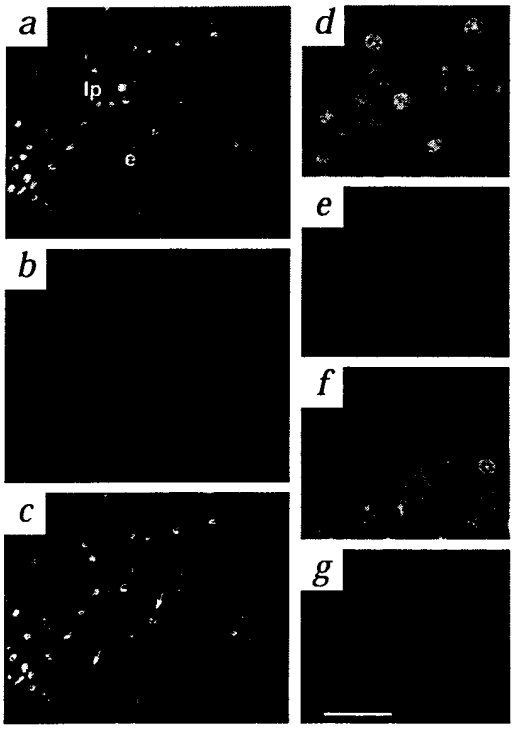

Fig. 4 *a*, Propidium iodide staining showing lamina propria (lp) and epithelial (e) cell layers from the proximal jejenum from a rat treated with AAVlac, 1 month after vector administration **[AUTHOR: OK?]**. *b*, Double-labelling with β-galactosidase immunohistochemistry and fluorescence detection showed β-gal immunoreactivity still localized mainly in the lamina propria and in various epithelial cells (arrows in *c*). *e*, A combination of the images from (*a*) and (*b*). Images (high magnification) of propidium iodide-stained lamina propria (*d*) and epithelial layer (*f*) from a control rat treated with AAVluc showed no corresponding β-galactosidase immunofluorescence (*e* and *g*). Scale bar represents 40 μm in *a–c* and 19 μm in *d–g*.

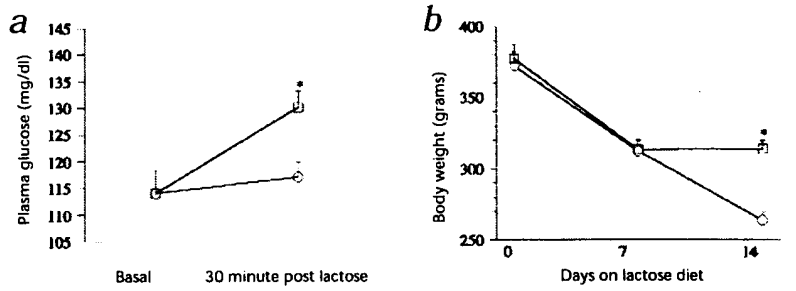

Fig. 5 Plasma glucose and body weight after an acute lactose challenge and a lactose-only diet. ***a***, Change in plasma glucose after the ingestion of lactose in rats fasted overnight. Rats were analyzed 1 week after AAVlac (⟶) or PBS (⟶) administration. *, $P = 0.013$, AAVlac compared with PBS for the 30-minute post-lactose, plasma glucose level (ANOVA with repeated measures and post-hoc Tukey's test). ***b***, Body weights at baseline and 1 week and 2 weeks after a 14-day lactose-and-water diet. The diet began 1 week after oral AAVlac (⟶) or PBS (⟶) treatment. At 2 weeks, the AAVlac group is significantly heavier than the PBS group (*, $P < 0.001$).

ability to metabolize lactose well enough to generate a significant rise in plasma glucose after an oral challenge. This result indicates that the β-galactosidase expression levels obtained were sufficient for efficient lactose metabolism. This is also consistent with the clinical use of adding exogenous β-galactosidase to lactose-containing milk to enable metabolism of the milk sugar. The other phenotypic effect of AAVlac administration was that rats maintained weight in the second week of a lactose-only diet whereas control rats continued to lose weight throughout the diet. Although both groups of rats ingested the lactose during this period (as demonstrated by the partial emptying of the dishes containing lactose), because of lactose spillage into the rat bedding we were unable to obtain accurate quantitative measures of lactose ingested for all rats. Thus, perhaps the control group, which continued to lose weight steadily throughout the 2-week period, simply did not eat the lactose. This is unlikely to explain the continued loss, as both groups were observed feeding over this period, with the control rats showing additional evidence of ingestion by partially emptying the lactose containers as well as by having the characteristic feces pellets that were not well formed with a high moisture content. Thus, despite the ingestion of the lactose diet, control rats probably were unable to metabolize the lactose consistent with a lactase deficiency state.

This vector and route of administration may have additional clinical potential beyond gut-enzyme gene therapy. The gut antigen presenting cells in the lamina propria effectively help generate systemic immune responses and are a target for vaccine development[20]. Oral AAV vectors may have potential for immunization similar to that of salmonella vectors[21]. The 6-month expression in the well vascularized gut lamina propria also indicates this route may be applicable for stable and persistent protein replacement, particularly when release into the portal circulation is desired. The 6-month duration of expression is consistent with AAV-mediated gene transfer in muscle, where a similar expression cassette led to transgene protein expression to 24 months[17].

Here we have demonstrated that a single peroral administration of an AAV vector can result in persistent expression and long-lasting phenotypic correction in a rodent model of hypolactasia. Our data indicate that a 'gene in a tablet' or a 'genetic pill' strategy using AAV vectors might be useful for a broad range of conditions. Moreover, the lack of toxicity and non-invasiveness of this approach should render oral AAV vectors as a palatable choice compared with current pharmacological treatments.

Methods

Animals. Rats used for the lactase-deficiency screening and lactose-challenge tests were obtained from Yale University School of Medicine, and studies were approved by the Animal Care and Use Committee. Additional rats used for the protein expression studies were obtained from the Animal Resources Unit, University of Auckland School of Medicine, and studies were approved by the Animal Ethics Committee.

Screening of lactase-deficient rats. Adult (more than 4 months old), male Fisher 344 rats were screened using an oral lactose challenge. Rats were fasted overnight. On the morning of the test, a baseline, fasting plasma glucose level was obtained from tail vein blood. The rat was then administered lactose orally, as a 25% lactose solution, and the plasma glucose was again measured in a tail-vein blood sample 30 minutes later. Plasma glucose was measured using a Beckman Glucose Analyzer II as described[22]. Rats with increase in plasma glucose of greater than 5 mg/dl were excluded from further study.

Generation of AAVlac. The AAV plasmid pAAVlac was created as described[18] from the parent plasmid pSub201. Recombinant AAVlac virus was generated using the helper-free packaging system described[16], with minor modifications. Both pAAVlac and the helper plasmid pAd/AAV (which contains both the *rep* and *capsid* opening reading frames of AAV flanked by adenovirus terminal repeats, but lacks the inverted terminal repeat of AAV) were transfected into 293 HEK cells using calcium phosphate. At 24 hours after transfection, the 293 cells were infected with adenovirus dl309 (obtained from T. Shenk, Princeton University, New Jersey) at an MOI of 5. At 48 hours after infection, the viruses (both dl 309 and recombinant AAV) were harvested using sonication. The crude extract was heat-inactivated and then purified using ammonium sulfate followed by double-cesium banding, and dialyzed against phosphate buffered saline and further concentrated. The control vector AAVluc (expressing luciferase) was packaged using the same protocol. All vector stocks were assayed for adenovirus con-

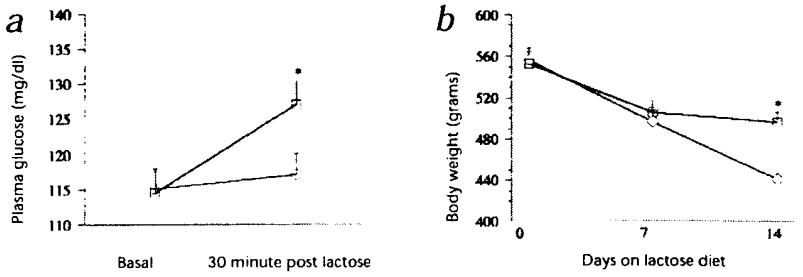

Fig. 6 ***a***, Change in plasma glucose after ingestion of lactose in rats fasted overnight, which were challenged 120 days after a single peroral dose of AAVlac (⟶) or PBS (⟶). *, $P = 0.03$. ***b***, Body weights at baseline and 1 week and 2 weeks after a 14-day lactose-and-water diet. The diet began 120 days after oral AAVlac (⟶) or PBS (⟶) treatment. *, $P = 0.002$.

tamination using a plaque assay on permissive 293 HEK cells, and were completely free of adenovirus.

Vector administration to lactase-deficient rats. Rats with flat glucose responses (less than 5 mg/dl change) to lactose were randomly assigned to 2 groups: AAVlac and PBS. After an overnight fast, rats were lightly anesthetized with ketamine/xylazine (60/6 mg/kg i.p.) and AAV vectors were administered via oral gavage. AAVlac was diluted with PBS to give 1.2×10^8 total infectious units in a final volume of 0.1 ml, and was orally administered to rats in the AAVlac group; control rats received 0.1 ml PBS alone. Rats were allowed to recover and returned to *ad libitum* access to water and a standard rat chow diet. The rats were housed in the Yale Animal Care Facility where they were kept on a 12-hour day–night cycle.

Lactose diet. Rat chow was removed from housing cages and bedding was replaced to ensure that no rat chow was present, and containers of lactose (Sigma) were placed into the cages with water available *ad libitum*. Rats were weighed at the beginning of and at 7 and 14 days after beginning the lactose diet. After 14 days, the lactose was removed and the rats were fed regular rat chow.

Lactose challenge test. Rats were fasted overnight and blood samples were obtained by nicking the tail vein. Rats were then given a 30-minute access to lactose (25% solution) in their home cages. A second tail vein sample was taken 30 minutes from the midpoint of the lactose meal. Blood was centrifuged immediately after collection, and the plasma was analyzed for glucose using a Beckman Glucose Analyzer II as described[22]. This relatively noninvasive approach was used because in preliminary studies we had determined that a forced oral dose of lactose using either an orogastric tube or liquid bottle feeding resulted in a highly variable stress hyperglycemic response that confounded interpretations of the data.

RT–PCR amplification of AAVlac mRNA. After peroral delivery of AAVlac, rats were killed and the guts, gonads, kidneys and lungs were removed and placed on dry ice immediately. The samples were stored at –80 °C before analysis. RNA from 100 mg of the proximal intestine and other organs was extracted using Trizol (Life Technologies). First-strand cDNA was synthesized using 5.0 μg of total RNA, which was primed with Oligo dt (0.5 μg; Promega), then reverse-transcribed using SuperScript II RNase H⁻ reverse transcriptase (150U; Life Technologies) at 42 °C for 90 min. Duplicate reactions without SuperScript II were negative controls. LacZ oligonucleotide primers LZ-1 (5'–GGTGCGGATTGAAAATGG–3') and LZ-2 (5'–GCTTCATCCACCACATAC–3') were used to generate a 219-bp product. Analysis of β-actin cDNA was an internal control for the PCR reactions. Primers for β-actin PCR were βA-1 (5–CTCTTCCAGCCTTCCTTCC–3') and βA-2 (5'–GTCACCTTCACCGTTCCAG–3'), which generated a 559-bp PCR product. Initial dilution and cycle series experiments were done to determine the range of cDNA input and number of PCR cycles for which the PCR amplification was linear for both PCR products. The cycling parameters were 5 min at 94 °C, followed by 40 cycles of 1 min at 94 °C, 1 min at 60 °C 1 min at 72 °C. After amplification, 5 μl of PCR products were electrophoresed on a 2% agarose gel containing ethidium bromide (Life Technologies) and visualized with UV light.

X-gal staining. For the X-gal staining and immunohistochemistry, at various time points after peroral administration of AAVlac or AAVluc, rats were overdosed with pentobarbital and perfused transcardially with saline followed by 2% paraformaldehyde containing 2 mM $MgCl_2$ and 1.25 mM EGTA in 0.1 M phosphate buffer (pH 8.0) to inhibit endogenous β-galactosidase[23]. The gastrointestinal tract was postfixed briefly before cryoprotection in 30% sucrose in PBS, and sections 16 μm in thickness were cut on a cryostat and thaw-mounted onto slides. Sections were immersed briefly in 4% paraformaldehyde, washed extensively with PBS and immersed in a solution containing 1 mg/ml X-gal, 2 mM $MgCl_2$, 5 mM $K_3Fe(CN)_6$ and 5 mM $K_4Fe(CN)_6$ in PBS overnight at 37 °C. Sections were rinsed in PBS and counterstained with hematoxylin/eosin and coverslipped in PBS/glycerol.

Immunohistochemistry. Sections were fixed for 15 min with 4% paraformaldehyde in 0.1 M phosphate buffer and rinsed with PBS containing 0.2% Triton-X100 (PBS–Triton) before incubation in 1% H_2O_2 in methanol for 2 min. After more PBS–Triton rinses, sections were incubated overnight at room temperature with a monoclonal antibody to β-galactosidase (1:25 dilution; Life Technologies). Sections were washed with PBS–Triton before a 2-hour incubation with secondary goat anti-mouse-Cy5 (Jackson Immunoresearch, West Grove, Pennsylvania). All antibodies were diluted in immunobuffer containing 1% normal goat serum and 0.4 mg/ml methiolate in PBS–Triton. After PBS–Triton washes, sections were incubated for 15 min with propidium iodide (5mg/ml; Sigma), rinsed in PBS and distilled water before being mounted with Vectashield (Vector Laboratories, Burlingame, California). Immunofluorescent signals were captured using a Leica 4d TCS confocal microscope and images were processed using Adobe Photoshop 4.0 (Adobe Systems, Mountain View, California).

Statistical analysis. Data are given as means ± s.e.m. and were analyzed using ANOVA with repeated measures and Tukey post-hoc tests using Systat (Evanston, Illinois) statistical software.

Acknowledgments

This work was supported by grants from the Juvenile Diabetes Foundation International, the New Zealand Health Research Council, New Zealand Marsden Fund and the New Zealand Lotteries Board.

RECEIVED 11 AUGUST; ACCEPTED 20 AUGUST 1998

1. Sahi, T., Isokoski, M., Jussila, J., Launiala, K. & Piorala, K. Recessive inheritance of adult-type lactose malabsorption. *Lancet* **2**, 823–826 (1973).
2. Lisker, R., Gonzalez, B. & Daltabuit, M. Recessive inheritance of the adult type of intestinal lactase deficiency. *Am. J. Hum. Genet.* **27**, 662–664 (1975).
3. Flatz, G. Genetics of lactose digestion in humans. *Adv. Human Genet.* **16**, 1–77 (1987).
4. Sandler, R.B. *et al.* Postmenopausal bone density and milk consumption in childhood and adolescence. *Am. J. Clin. Nutr.* **42**, 270–274 (1985).
5. Birge, S.J., Keutmann, H.T., Cuatrecasas, P. & Whedon, G.D. Osteoporosis, intestinal deficiency and low dietarty calcium intake. *New Engl. J. Med.* **276**, 445–448 (1967).
6. Honkanen, R. *et al.* Lactose intolerance associated with fractures of weight-bearing bones in Finnish women aged 38–57 years. *Bone* **21**, 473–477 (1997).
7. Stallings, V.A., Oddleifson, N.W, Negrini, B.Y., Zemel, B.S. & Wellens, R. Bone mineral content and dietary calcium intake in children prescribed a low-lactose diet. *J. Ped. Gastroenterol. Nutr.* **18**, 440–5 (1994).
8. Buller, H.A. *et al.* New insights into the lactase and glycosylceramindase adctivities of rat microvillus membrane lactase-phlorizin hydrolase. *Am. J. Physiol.* **257**, G616–G623 (1989).
9. Scrimshaw, N.S. & Murray, E.B. The acceptability of milk and milk products in populations with a high prevalence of lactose intolerance. *Am. J. Clin. Nutr.* **48**, 1083–1159 (1988).
10. Montgomery, R.K., Buller, H.A., Rings, E.H. & Grand, R.J. Lactose intolerance and the genetic regulation of intestinal lactase-phlorizin hydrolase. *FASEB J.* **5**, 2824–32 (1991).
11. Witte, J., Lloyd, M., Lorenzsonn, V., Korsmo, H. & Olsen, W. The biosynthetic basis of adult lactase deficiency. *J. Clin. Invest.* **86**, 1338–42 (1990).
12. Maiuri, L. *et al.* Mosaic regulation of lactase in human adult-type hypolactasia. *Gastroenterology* **107**, 54–60 (1994).
13. Sebastio, G. *et al.* The control of lactase in human adult-type hypolactasia and in weaning rabbits and rats. *Am. J. Human Genet.* **45**, 489–497 (1989).
14. Sterchi, E.E. *et al.* Biogenesis of intestinal lactase-phlorizin hydrolase in adults with lactose intolerance. Evidence for reduced biosynthesis and slowed-down maturation in enterocytes. *J. Clin. Invest.* **86**, 1329–37 (1990).
15. Berns, K.I. & Hauswirth, W.W. Adeno-associated viruses. *Adv. Virus Res.* **25**, 407–409 (1979).
16. Samulski, R.J., Chang. L-S. & Shenk, T. Helper-free stocks of adeno-associated viruses: normal integration does not require viral gene expression. *J. Virol.* **63**, 3822–3828 (1989).
17. Xiao, X., Li, J. & Samulski, R.J. Efficient long-term gene transfer into muscle tissue of immuno-competent mice by adeno-associated virus vector. *J. Virol.* **70**, 8098–108 (1996).
18. Kaplitt, M.G *et al.* Long-term gene expression and phenotypic correction using adeno-associated virus vectors in the mammalian brain. *Nature Genet.* **8**, 148–154 (1994).
19. Trier, J.S. Structure and function of intestinal M cells. *Gastroenterol. Clin. North Am.* **20**, 531–547 (1991).
20. Service, R.F. Triggering the first line of defense. *Science* **265**, 1522–1524 (1994).
21. Darji A. *et al.* Oral somatic transgene vaccination using attenuated S. typhimurium. *Cell.* **91**, 765–75 (1997).
22. During, M.J. *et al.* Glucose modulates rat substantia nigra GABA release in the freely-moving rat via an effect on ATP-sensitive potassium channels. *J. Clin. Invest.* **95**, 2403–2408 (1995).
23. Kaplitt, M.G. *et al.* Preproenkephalin promoter yields region-specific and long-term expression in adult brain following direct in vivo gene transfer via a defective herpes simplex viral vector. *Proc. Natl. Acad. Sci. USA* **91**, 8979–8983 (1994).